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Metallic elements in Nile Crocodile eggs from the Kruger National Park, South Africa

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ABSTRACT

The Nile Crocodile is the largest predator on the African continent. Recent mass mortalities in the Kruger National Park (KNP) raised concerns about possible influence of pollution. We analysed eggs and their eggshells collected from nests inside the KNP and from a crocodile farm for metallic elements. We found that mercury, selenium, and copper occurred at levels of concern. Eggshells had very high concentrations of iron. Apart from toxicological implications associated with elevated concentrations in eggs, we found iron possibly contributing towards thicker eggshells. Thicker shells may act as a barrier to gas and water exchange, as well as possibly increasing the effort required for the hatchling to emerge from tightly packed shells under sand. Pollutants are transported into the KNP via rivers, and possibly via air. Mercury and copper pollution are waste-, industrial- and mining-related; ecotoxicological concern should therefore be extended to all areas where the four African crocodile species occur. Reptiles are under-represented in ecotoxicological literature in general, and especially from Africa. We know of only one previous report on metals and metalloids in crocodile eggs from Africa (Zimbabwe), published 30 years ago. Reduced fitness, endocrine disruption and effects on behaviour are other possible sub-lethal effects associated with metallic elements that may only become apparent decades later in a long-lived species such as the Nile Crocodile. In the face of habitat destruction, pollution, human population increases, and climate change, further research is needed regarding pollutant concentrations and effects in all African reptiles. The rivers that carry water from outside the park sustain its aquatic life, but also transport pollutants into the KNP. Therefore, improved source mitigation remains an important task and responsibility for all involved.

1. Introduction

The Nile Crocodile *Crocodylus nyloticus* is ranked as Least Concern by the International Union for Conservation of Nature (IUCN) (IUCN, 2016). In spite of this, their numbers are declining alarmingly in some areas in South Africa (Botha et al., 2011; Ferreira and Pienaar, 2011; Downs et al., 2015) and Botswana (Bourquin and Leslie, 2011). Reasons advanced for this decline are loss of breeding habitat (Leslie and Spotila, 2001), exploitation (Bourquin and Leslie, 2011), pollution (Botha et al., 2011), and disease (Ferreira and Pienaar, 2011). Adding to the concerns, mass crocodile deaths occurred at the confluence of the Letaba and Olifants rivers situated within the Kruger National Park (KNP; Fig. 1) as well as some deaths in the Sabie River further south in the KNP. These deaths were caused by or associated with pansteatitis, a condition where the fat becomes hardened, inflamed, and yellow (Osthoff et al., 2010). These incidents precipitated much research to elucidate the cause of the deaths and possible associated pollution (Ashton, 2010; Osthoff et al., 2010; Ferreira and Pienaar, 2011; Woodborne et al., 2012; Bouwman et al., 2014; Du Preez et al., 2016; Gerber et al., 2017). Some suspected causes and contributing factors include:

- Microcystins from cyanobacteria (Myburgh and Botha, 2009).
- Pollutants settling out of the water as the river slow down entering the Massingir Dam in Mozambique (Osthoff et al., 2010).
- Crocodiles consuming rancid fish (Ashton, 2010; Huchzermeyer et al., 2011).
- Environmental decline and pollution (Ferreira and Pienaar, 2011).
- Crocodiles feeding on steatitic African Sharp-toothed Catfish *Clarias gariepinus* (Huchzermeyer et al., 2011).
- Ecosystem changes combined with extra-limital fish species as vector of the cause (Woodborne et al., 2012).

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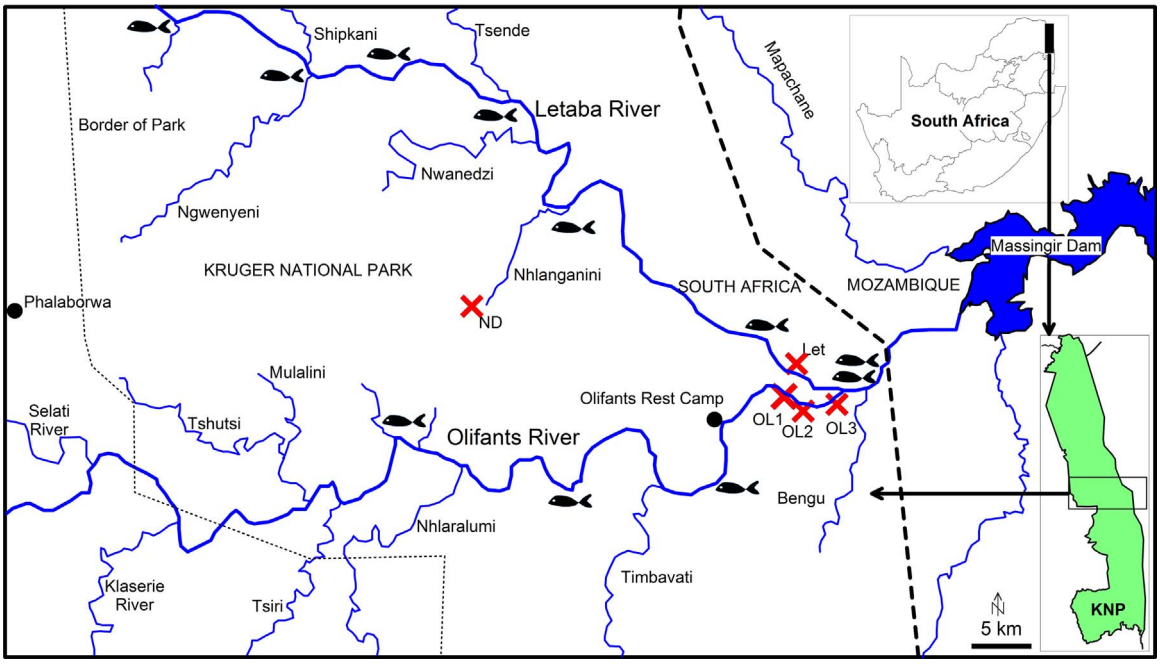

Fig. 1. Map of locations in the Kruger National Park where crocodile eggs were collected, marked with Xs. Sites with fish are recognised national fish sanctuaries. Rivers flow from east to west.

- High concentrations of aluminium in the fat of the Nile tilapia *Oreochromus mossambicus* that may interfere with cellular metabolism such as lipid-peroxidation (Oberholster et al., 2012).
- Seasonal change in diet due to potamodromic migrations of the invasive Silver Carp *Hypophthalmichthys molitrix* that has a fatty acid composition different from indigenous fish (Huchzermeyer, 2012).

Crocodilians are large, amphibious, and apex predators that may be considered bio-indicators because they accumulate a wide range of contaminants over long lifespans (Bouwman et al., 2014; Nifong et al., 2014). In Africa in freshwater ecosystems where they occur, the Nile Crocodile is the apex predator (Nifong et al., 2014). Their trophic levels are similar to sharks in marine ecosystems and polar bears in the Arctic. Total concentrations of contaminants in water and sediments however, do not always reflect their bio-availability (Pheiffer et al., 2014). It is therefore, difficult to assess if the concentrations in water or sediment might cause biological harm (Pheiffer et al., 2014), especially in long-lived animals such as crocodiles. Comparing results with other studies will therefore, assist in the identification of risk associated with the pollutants measured (Cortes-Gomez et al., 2014).

Eggs are often used in bio-monitoring (Klein et al., 2012). Eggs (avian or reptilian) represent contaminant levels within the female as well as the newly developing embryo (Klein et al., 2012; Bouwman et al., 2014; Cortes-Gomez et al., 2014; van der Schyff et al., 2016). Not only the contents, but also the shell may have toxicological implications when considering the distribution of pollutants between shell and contents (Kleinow et al., 1997). Another advantage of using eggs is that no reproducing adult animals have to be captured or killed. Collecting eggs from some species however, are often difficult (and hazardous), especially crocodiles (Bouwman et al., 2014).

According to an assessment of emerging chemical management issues in developing countries that are not covered by international treaties, “heavy metal” pollution is ranked the highest of the 22 issues identified (STAP, 2012). Geology plays an important role in the natural background of metals and metalloids, although anthropogenic activities such as agriculture, mining, and wastes handling may increase bio-available and bio-accessible metals in excess of background (Luoma, 1983). Therefore, elemental concentrations in biota are derived from both natural background and pollution, where pollution has occurred. Some metals like lead, cadmium, arsenic, and mercury have adverse effects on biota, while iron, magnesium, copper, and zinc have important physiological functions (Birch and Taylor, 1999; Hoekstra et al., 2003; Peijnenburg and de Jager, 2003; Grillitsch and Schiesari, 2010). Most metals and metalloids can be toxic at elevated concentrations, some even at relatively low concentrations (Zhou et al., 2008).

We could find only one study reporting metal concentrations in eggs of any of the four species of crocodile occurring in Africa. Phelps et al. (1986) reported on organic micro pollutants and mercury, selenium, cadmium, lead, and zinc in 26 Nile Crocodile eggs from ten sites in Zimbabwe. Organic micro pollutants have been reported in Nile Crocodile eggs from the Kruger National Park, South Africa (Bouwman et al., 2014). Here, we report the findings and interpretation of metallic elements in the same eggs collected in the KNP that were published by Bouwman et al. (2014) for POPs, The collection sites were mainly close to where the crocodile mortalities occurred. We also analysed the corresponding eggshells for the same elements to determine if the shells could serve as a non-lethal bio-monitoring method instead of using egg contents. Using the shells after hatching prevents the destruction of live embryos and may provide important information.

2. Materials and methods

2.1. Description of the Kruger National Park and sampling sites

The Kruger National Park (KNP) has six major river systems. The Letaba and Olifants rivers (Fig. 1) were where the crocodile mortalities occurred. The Letaba River (flowing to the east) confluences with the Olifants River, thereafter running through a gorge known as the Olifants Gorge (Fig. 1), below called the Gorge. The water of the Olifants River is highly mineralized (Du Preez and Steyn, 1992). The Olifants River is a system in which water quality is affected by human activities to a greater degree than from geological background (Gerber et al., 2015a). Metal bio-accumulation studies that were done in the Olifants River on African Sharp-toothed Catfish (*C. gariepinus*) indicated that fish inside the basin of the KNP had lower concentrations of metals than those collected upstream of the KNP (Du Preez et al., 1997). The Letaba

River is experiencing flow restrictions and increased sediment build up due to the large number of dams and weirs built for agricultural and industrial purposes. The section upstream from the border of the KNP is considered the most modified section of the whole Letaba River system (DWAF, 2006). Outside the Park, the Letaba and Olifants river systems are influenced by anthropogenic activities such as mining, power generation, many industries, agriculture, and urbanisation (Coetzee et al., 2002; Dabrowski and de Klerk, 2013). The Nhlanganini catchment however, is entirely located within the KNP - therefore the minerals in water and sediments here should be ascribed to hydrology, geology, aerial inputs, and/or animals, and serves as a reference (Bouwman et al., 2014). We sampled the eggs from the shores of the small, cement-walled Nhlanganini Dam (built in 1968), upstream of which there are no anthropogenic activities (Fig. 1).

2.2. Collection

All the eggs were collected between October and December 2009 under the supervision of SANParks (South African National Parks) veterinarians and wildlife experts, under permit and with permissions. Ethical approval was granted by the North-West University (NWU-00055-07-S3). The crocodile nests were located by field rangers from a helicopter looking for patches of newly covered nests along the riverbanks, and marked potential nests with a global positioning system (Fig. 1). A ground team then located the nests and collected the eggs by digging with their hands and removing eggs from nests – no more than six eggs were collected per nest. After collection, all dug-up sand was replaced and signs of disturbance were removed to minimize opportunistic predation.

Twenty-seven eggs were collected from five nests in the KNP and a crocodile farm In the KNP, three nests from the Olifants River OR1 (n = 3 eggs), OR2 (n = 3 eggs) and OR3 (n = 3 eggs), collectively named Gorge, were sampled. One nest from the Letaba River Let (n = 6 eggs) and one nest at Nhlanganini Dam (ND, n = 2 eggs) (Fig. 1) were also sampled. Ten, randomly selected eggs were collected from a hatchery of a large crocodile farm (Crocodile Farm) situated south of the KNP. Eggs were wrapped in foil (cleaned with acetone and hexane beforehand), labelled, and frozen.

2.3. Sample preparation and analyses

At the laboratory, frozen eggs were placed in pre-washed (acetone and hexane) foil cups and left overnight to thaw. Thawed eggs were washed with deionized water to remove sand, circumferences measured, and weighed. Egg contents (yolk and albumin together) were then separated from the shells. Wet mass of the contents was determined and sub-samples placed in clean polyethylene bottles. The sub-samples were freeze-dried (48 h at −80 °C and 0.13 Pa) and then powdered. The eggshells were washed with water to remove the shell membrane, and air-dried for at least three weeks. The dried shells were then ground with a mortar and pestle into a powder.

Powdered samples were analysed by Eco-Analytica in Potchefstroom, South Africa. Eco-Analytica analysed the powered products for 26 metals and metalloids using the EPA 3050b method (EPA, 1996). The method used two grams of powdered sample. The laboratory regularly participates in inter-laboratory calibration exercises, and a certified standard reference material was used (SRM 1944). Concentrations were within 25% of certified values. Concentrations are expressed in mg/kg dry mass (dm).

The inner and outer layers of the shell separate very easily. We mounted three fragments of each layer per egg on stubs for scanning electron microscopy. The thickness of each fragment was measured at five different points.

2.4. Statistical analyses

We analysed data using Prism 7.02 (www.graphpad.com) and PC-ORD 7.02 (MjM Software, www.pcord.com). In most cases, except where indicated, data were log-transformed. Significance was taken at $p < 0.05$. One-way ANOVA (Kruskal-Wallis test, with Dunn's multiple comparisons test) was used to look at differences between collection sites (Fig. 1) for both contents and eggshells, separately. We used paired, two-way, *t*-tests to compare differences of the mean elemental concentrations of shell and content. We used linear regressions to investigate the relationships between the concentrations of the same element between shells and contents.

The percentage coefficients of variation (%CV) was used to identify elements with large and small absolute variations. We used the Wilcoxon matched-pairs, signed rank test (non-parametric) to compare untransformed %CV values because the shell and contents of the same egg are essentially paired. The Pearson correlation coefficient was used to test the effectiveness of pairing (the more effective the pairing between the values, the smaller the p-value will be). Linear regressions were used to investigate the relationship between the same elements in eggs and shells using both untransformed and log-transformed concentrations.

We used a multi-response permutation procedure (MRPP) to compare elemental profiles (fingerprints) between shells and contents, with concentrations relativized per egg, was used with Euclidian as distance measure. The *T*-statistic describes the separation between the shells and contents – the more negative the value, the stronger the separation between the groups. MRPP also calculates a chance-corrected within-group agreement (A = agreement value between 0 and 1; when all values are identical between groups A = 1; when heterogeneity within groups equals expectation by chance A = 0), as well as the probability of a smaller or equal difference in elemental concentration profile (p –value).

To visualise the differences in relative elemental composition profiles, we used non-metric multidimensional scaling (NMS), again with Euclidian as distance measure. Six dimensions were allowed, with a maximum of 500 iterations. Two-hundred and fifty runs with real data were allowed to obtain the final stress, followed by the same number with randomised data for a Monte Carlo test to determine if a similar final stress could have been reached by chance (McCune and Grace, 2002). Because of concentrations orders of magnitude higher than the other elements, iron was not included in the NMS and MRPP. Because aluminium was used to wrap the eggs, this element was not used in comparative statistics. We use convex hulls (polygons that enclose all outermost points of each specific group) to discern difference (no overlap) or congruence (overlap).

3. Results

3.1. Analytical results

The mean concentrations (mg/kg dm) of seventeen metals and metalloids in the shells and egg contents of crocodile eggs from four different collection areas are presented in Table 1. Additional summary statistics are available for all elements tested (Supplemental materials, Table S1). Although the eggs were wrapped in aluminium (Al) foil upon collection, the Al data is still provided here for reference, but not used in further statistics, as we cannot exclude contamination of shell and contents from the foil.

3.2. Comparisons between sites

Concentrations of metals and metalloids differed significantly between the different collection areas (one-way ANOVA Kruskal-Wallis test, with Dunn's multiple comparisons test - $p < 0.05$) for 22 elements in eggshells, and 12 in egg contents (Table 2, and further data in Table

Table 1
Mean concentrations of selected elements in crocodile egg contents and shells, compared with the results from other studies. Additional and expanded results are provided in Supplemental materials, Table S1.

Species	Sample	Year	Matrix	Locality	Al	Cu	Hg	Pb	Cr	Ni	Co	Cd	Zn	Se	As	Mn	Fe	Ba	V	Pt	Au	Ti	Ag	Reference
Nile Crocodile	**Dry**	**2009**	**Content**	**Crocodile Farm, South Africa***	**19**	**8.6**	**1.1**	**0.80**	**1.4**	**0.52**	**0.049**	**0.037**	**18**	**4.0**	**0.95**	**1.9**	**61**	**8.5**	**0.50**	**0.047**	**0.063**	**7.0**	**0.11**	**This Study**
Nile Crocodile	**Dry**	**2009**	**Content**	**Letaba River KNP, South Africa**	**20**	**6.6**	**0.90**	**0.58**	**1.4**	**0.48**	**0.048**	**0.020**	**12**	**3.7**	**0.77**	**1.7**	**55**	**10**	**0.48**	**0.021**	**0.23**	**6.8**	**0.073**	**This Study**
Nile Crocodile	**Dry**	**2009**	**Content**	**Gorge, KNP South Africa**	**16**	**7.7**	**0.76**	**0.76**	**1.5**	**0.48**	**0.045**	**0.018**	**14**	**4.6**	**0.74**	**1.7**	**52**	**7.8**	**0.51**	**0.063**	**0.15**	**6.9**	**0.13**	**This Study**
Nile Crocodile	**Dry**	**2009**	**Content**	**Nhlanganini, KNP, South Africa**	**19**	**9.6**	**0.63**	**1.5**	**1.5**	**0.44**	**0.049**		**15**	**3.8**	**0.71**	**1.6**	**52**	**7.2**	**0.55**	**0.016**	**0.15**	**6.8**	**0.32**	**This Study**
Nile Crocodile	Dry	1981	Content	Sengwa River, Zimbabwe			0.46	2.6				0.07	34	0.83										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Mpalangrna River, Zimbabwe			0.093	2.8				0.084	43	0.84										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Zambezi River, Zimbabwe			0.067	2.1				0.074	46	0.7										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Zimbabwe, Kariba Crocodile farm*			0.12	3.9				0	38	0.71										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Lake Mcliwaine, Zimbabwe			0.2	3.2				0.063	26	0.98										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Ngewi River, Zimbabwe			0.21	7.8				0.15	34	0.56										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Zimbabwe, Kyle Recreational Park*			0.2	8.6				0.073	32	0.89										Phelps et al. 1986
Nile Crocodile	Dry	1981	Content	Runda River, Zimbabwe			0.093	2.4				0	37	1.08										Phelps et al. 1986
American Crocodile	Dry	1980	Content	Florida Bay, USA	11	6.2	0.66	3.4	2.6	2.35	1.12	0.13												Stoneburner and Kushlan (1984)
American Crocodile	Wet	1995	Content	Sapote Lagoon, Belize			0.11																	Rainwater et al. (2007)
American Crocodile	Wet	1995	Content	Gold Button Lagoon, Belize			0.11																	Rainwater et al. (2007)
American Crocodile	Wet	1995	Content	Laguna Seca, Belize			0.21																	Rainwater et al. (2007)
American Alligator	Wet	1971	Content	Shark valley, USA			0.54								0.13									Ogden et al. (1975)
American Alligator	Wet	1984	Content	Lake Okeechobee, USA	1.5	0.32		0.14	0.09	0.07			6.7	0.31		0.14	13							Heinz et al. (1991)
American Alligator	Wet	1984	Content	Lake Griffin, USA	2	0.78		0.22	0.08	0.05			7.6	0.37		0.15	13							Heinz et al. (1991)
Chinese Alligator	Dry	2003	Content	Changxing Breeding Center, China*		33	0.11	0.8	0.098			0.17	59		0.47	1.7								Xu et al. (2006)
Chinese Alligator	Dry		Content	Anhui Province, China		2.2		0.73				0.056	6.2											Ding et al. (2001)
Chinese Alligator	Dry		Content	Anhui Captive Breeding Center, China*		2.5		1.2				0.1	10											Ding et al. (2001)
American Crocodile	Wet	1971	Content	Florida Bay, USA		3.7	0.09	0.34				0.05	11		0.072									Ogden et al. (1975)
American Alligator	Wet	1984	Content	Lake Apopka, USA	1.3	0.52			0.09	0.09			5.6	0.3		0.14	11							Heinz et al. (1991)
Mediterranean Chameleon	Wet	2001	Content	Southwest Spain		0.6		9.0				0.087	12		0.004									Gómara et al. (2007)
Pond Slider Turtles	Dry	1996	Content	South Carolina, USA			0.040	687	0.14			0.067		0.42	0.004									Burger and Gibbons (1998)
Western Pond Turtle	Wet	1997	Content	Oregon, USA		0.0				0.002			0.065			2.2	89							Henny et al. (2003)
Nile Crocodile	**Dry**	**2009**	**Shell**	**Crocodile Farm, South Africa***	**7.6**	**7.3**	**0.34**	**1.2**	**2.3**	**6.5**	**0.29**	**0.015**	**3.7**	**1.7**	**2.1**	**1.7**	**1700**	**36**	**0.88**	**0.024**	**0.036**	**0.29**	**0.069**	**This Study**
Nile Crocodile	**Dry**	**2009**	**Shell**	**Letaba River KNP, South Africa**	**2.1**	**8.0**	**0.37**	**0.62**	**2.5**	**6.6**	**0.27**	**0.0033**	**3.2**	**1.7**	**2.5**	**1.7**	**1700**	**52**	**0.90**	**0.023**	**0.026**	**0.19**	**0.062**	**This Study**
Nile Crocodile	**Dry**	**2009**	**Shell**	**Gorge, KNP South Africa**	**16**	**3.9**	**0.24**	**0.66**	**2.5**	**7.8**	**0.32**	**0.0022**	**2.1**	**1.6**	**2.8**	**1.6**	**1900**	**30**	**0.94**	**0.040**	**0.013**	**0.25**	**0.044**	**This Study**

(*continued on next page*)

Table 1 (*continued*)

Species	Sample	Year	Matrix	Locality	Al	Cu	Hg	Pb	Cr	Ni	Co	Cd	Zn	Se	As	Mn	Fe	Ba	V	Pt	Au	Ti	Ag	Reference
Nile Crocodile	**Dry**	**2009**	**Shell**	**Nhlanganini, KNP, South Africa**	**43**	**7.3**	**0.33**	**0.46**	**2.8**	**5.6**	**0.21**		**4.0**	**1.6**	**2.3**	**1.3**	**1300**	**20**	**1.01**	**0.103**	**0.024**	**0.69**	**0.070**	**This Study**
Chinese Alligator	Dry	2003	Shell	Changxing Breeding Center, China*			44	1.2	0.31			0.23	9		0.26	14	46							Xu et al. (2006)
Chinese Alligator	Dry		Shell	Anhui Captive Breeding Center, China*		3.7		26				3.4	38											Ding et al. (2001)
Chinese Alligator	Dry		Shell	Anhui Province, China		2.2		15				1.8	24											Ding et al. (2001)
American Crocodile	Dry	1980	Shell	Florida Bay, USA	52	17	0.21	21	20	22.04	1.7	0.36												Stoneburner and Kushlan (1984)
Mediterranean Chameleon	Dry	2001	Shell	Southwest Spain				0.72				0.066	8.7		0.073									Gómara et al. (2007)
Pond Slider Turtles	Dry	1996	Shell	South Carolina, USA			0.22		0.38			0.013	0.036		0.003									Burger and Gibbons (1998)

S1). The egg contents from the Crocodile Farm had significantly higher concentrations of arsenic (As), lead (Pb), mercury (Hg), and thallium (Tl) when compared with the Gorge eggs, and higher copper (Cu), As, molybdenum (Mo), and Tl concentrations when compared with Letaba. Only boron (B) and chrome (Cr) in egg content from the Crocodile Farm had significantly lower concentrations compared with Gorge (Table S1). The only element that differed significantly between the eggs from Letaba and Gorge was barium (Ba), with higher concentrations in Letaba. The Crocodile Farm shells had significantly higher concentrations of B, zinc (Zn), Mo, uranium (U), Hg, and gold (Au), compared with the Gorge, and significantly higher concentrations of Pb compared with Letaba. The concentrations of metallic elements in the Letaba eggshells had significantly lower concentrations of nickel (Ni), Cu, silver (Ag), Ba, and Au compared with the Gorge. The Gorge eggs had significantly higher concentrations of cobalt (Co), platinum (Pt), and iron (Fe) compared with Letaba.

3.3. Comparison between egg contents and eggshells

Egg contents (dm) had significantly higher (one-way ANOVA, Kruskal-Wallis test, with Dunn's multiple comparisons test - $p < 0.05$) Zn, Hg, Au, selenium (Se) and titanium (Ti) concentrations than shells. The eggshells on the other hand, had higher concentrations of Ni, Co, Ba, As, Pb, Cr, and Fe (Table 1 and S1).

Comparisons of mean %CVs showed that shell concentrations were more variable (means and medians of %CVs) compared with egg contents (Table 3). The lowest %CV was 0.9 for V in shell and 3.3 for Cr in contents, while the largest %CVs were U (263) in shells and Au (215) in contents (Table 3). The Wilcoxon matched-pairs, signed rank test showed a marginal significant difference ($p = 0.0825$), while pairing was highly significant ($p < 0.0001$; Spearman correlation coefficient, $rs = 0.5572$) showing that in general, if an element was highly variable in contents, it also tended to be highly variable in shells, and vice versa.

The results of linear regressions of untransformed and log-transformed elemental concentrations in eggs and shells are presented in Table S2, and some of the regressions are shown in Fig. 2. Only five elements had significant relationships; two of them (Co and As) were negative, and three (B, Mo, and Ba) were positive. The regressions of Cu, Zn, As, Pt, Hg, and Se are shown in Fig. 2a-f. Fig. 2g shows the regressions between Fe and the thickness of the inner and outer shells of the egg, and Fig. 2h the regression of the Se and Hg molar concentrations in contents.

3.4. Multivariate analyses

The MRPP showed a strong separation between the %CVs of the shells and contents ($T = -31$), and low agreement (high heterogeneity) between variations in shells and contents ($A = 0.3598$) (Table 3). The pairing was effective though, with the same elements tending to have correspondingly higher or lower %CVs in both contents and shells.

The NMS ordination plot (Fig. 3) required only two axes and 54 iterations to reach a stable (final instability = 0.0000) and low stress (3.828) solution. A final stress value < 5 shows an excellent representation of the relationships between samples (McCune and Grace, 2002). Axis 1 explains 91.8% of the variation, and axis 2 only 6.9% (Fig. 3). Egg contents had higher relative proportions of metals such Ti, Zn, Au, Hg, Ag, Se, B, etc. Eggshells had higher relative proportions of Rh, Ni, Co, Ba, As, etc. Metals such as Sn, Cd, Pb, and Mn had similar relative proportions in both shells and contents. Also clear from Fig. 2 is that the relative elemental proportions of the elements overlapped for contents, and separately overlapped for shells.

Table 2
Comparisons of %CVs of elemental concentrations in eggshells and –contents, as well as the results of the multi-response permutation procedure (MRPP) pattern analyses of 'fingerprints', based on relativized data. The *T*-statistic describes the separation between the shells and contents – the more negative the value, the stronger the separation between the groups. MRPP also calculates a chance-corrected within-group agreement (A = agreement value between 0 and 1; when all values are identical between groups A = 1; when heterogeneity within groups equals expectation by chance A = 0), as well as the probability of a smaller or equal difference in elemental concentration profile (p –value).

Egg contents						Eggshells				
Element	p	CF vs. Let	CF vs. Gorge	Let vs. Gorge	Number Sig	p	CF vs Let	CF vs Gorge	Let vs Gorge	Number Sig
B	**< 0.0001**	ns	***	ns	1	**0.0029**	ns	**	ns	1
Al	0.422	ns	ns	ns	0	**0.0244**	ns	ns	*	1
Ti	0.6711	ns	ns	ns	0	0.6223	ns	ns	ns	0
V	0.0703	ns	ns	ns	0	**0.0007**	ns	***	ns	1
Cr	**0.0173**	ns	*	ns	1	**0.0004**	ns	***	ns	1
Mn	0.7507	ns	ns	ns	0	0.2027	ns	ns	ns	0
Co	0.9529	ns	ns	ns	0	**0.0339**	ns	ns	*	1
Ni	0.7582	ns	ns	ns	0	**0.0014**	ns	**	*	2
Cu	**0.0409**	*	ns	ns	1	**0.0327**	ns	ns	*	1
Zn	0.2383	ns	ns	ns	0	**0.0003**	ns	***	*	2
As	**0.0015**	*	**	ns	2	**0.0002**	ns	***	ns	1
Se	0.1605	ns	ns	ns	0	0.145	ns	ns	ns	0
Mo	**0.0053**	**	ns	ns	1	**0.0009**	ns	***	ns	1
Rh	0.7659	ns	ns	ns	0	**0.0002**	**	**	ns	2
Pd	**0.0224**	ns	*	ns	1	**0.0432**	ns	ns	ns	0
Ag	0.1413	ns	ns	ns	0	**0.0244**	ns	ns	*	1
Sn	0.5941	ns	ns	ns	0	**0.0343**	ns	*	ns	1
Ba	**0.0059**	ns	ns	**	1	**0.0046**	ns	ns	**	1
Pt	**0.0936**	ns	ns	ns	0	0.5801	ns	ns	ns	0
Au	**0.0402**	ns	ns	ns	0	**0.0045**	ns	**	*	2
Hg	**0.0363**	ns	*	ns	1	**0.0002**	ns	**	***	2
Tl	**0.0008**	*	**	ns	2	**0.0402**	ns	ns	ns	0
Pb	**0.0224**	ns	*	ns	1	**0.0432**	ns	ns	ns	0
Fe	0.3669	ns	ns	ns	0	**0.0162**	ns	ns	*	1
U	**0.0537**	ns	ns	ns	0	**0.0041**	ns	**	ns	1
Number Sig	13	4	7	1	12	21	1	12	10	23

4. Discussion

4.1. Elemental concentrations

Scrutinizing the means per element in contents and shells from the KNP sites (Table 1) shows remarkably similar concentrations between sites. Of the 288 ANOVA post-tests between eggs from the four collection areas (Crocodile Farm, Letaba, Gorge, and Nhlanganini Dam) and 27 elements (Section 2.3), there were 14 significant differences for egg contents, and 23 for eggshells. There were no differences between the

Table 3
Results of linear regressions between elemental concentrations in eggshells and –contents, both for untransformed and transformed data.

Eggs						Shells				
Element	p	CF vs. Let	CF vs. Gorge	Let vs. Gorge	Number Sig	p	CF vs Let	CF vs Gorge	Let vs Gorge	Number Sig
B	**< 0.0001**	ns	***	ns	1	**0.0029**	ns	**	ns	1
Al	0.422	ns	ns	ns	0	**0.0244**	ns	ns	*	1
Ti	0.6711	ns	ns	ns	0	0.6223	ns	ns	ns	0
V	0.0703	ns	ns	ns	0	**0.0007**	ns	***	ns	1
Cr	**0.0173**	ns	*	ns	1	**0.0004**	ns	***	ns	1
Mn	0.7507	ns	ns	ns	0	0.2027	ns	ns	ns	0
Co	0.9529	ns	ns	ns	0	**0.0339**	ns	ns	*	1
Ni	0.7582	ns	ns	ns	0	**0.0014**	ns	**	*	2
Cu	**0.0409**	*	ns	ns	1	**0.0327**	ns	ns	*	1
Zn	0.2383	ns	ns	ns	0	**0.0003**	ns	***	*	2
As	**0.0015**	*	**	ns	2	**0.0002**	ns	***	ns	1
Se	0.1605	ns	ns	ns	0	0.145	ns	ns	ns	0
Mo	**0.0053**	**	ns	ns	1	**0.0009**	ns	***	ns	1
Rh	0.7659	ns	ns	ns	0	**0.0002**	**	**	ns	2
Pd	**0.0224**	ns	*	ns	1	**0.0432**	ns	ns	ns	0
Ag	0.1413	ns	ns	ns	0	**0.0244**	ns	ns	*	1
Sn	0.5941	ns	ns	ns	0	**0.0343**	ns	*	ns	1
Ba	**0.0059**	ns	ns	**	1	**0.0046**	ns	ns	**	1
Pt	**0.0936**	ns	ns	ns	0	0.5801	ns	ns	ns	0
Au	**0.0402**	ns	ns	ns	0	**0.0045**	ns	**	*	2
Hg	**0.0363**	ns	*	ns	1	**0.0002**	ns	**	***	2
Tl	**0.0008**	*	**	ns	2	**0.0402**	ns	ns	ns	0
Pb	**0.0224**	ns	*	ns	1	**0.0432**	ns	ns	ns	0
Fe	0.3669	ns	ns	ns	0	**0.0162**	ns	ns	*	1
U	**0.0537**	ns	ns	ns	0	**0.0041**	ns	**	ns	1
Number Sig	13	4	7	1	12	21	1	12	10	23

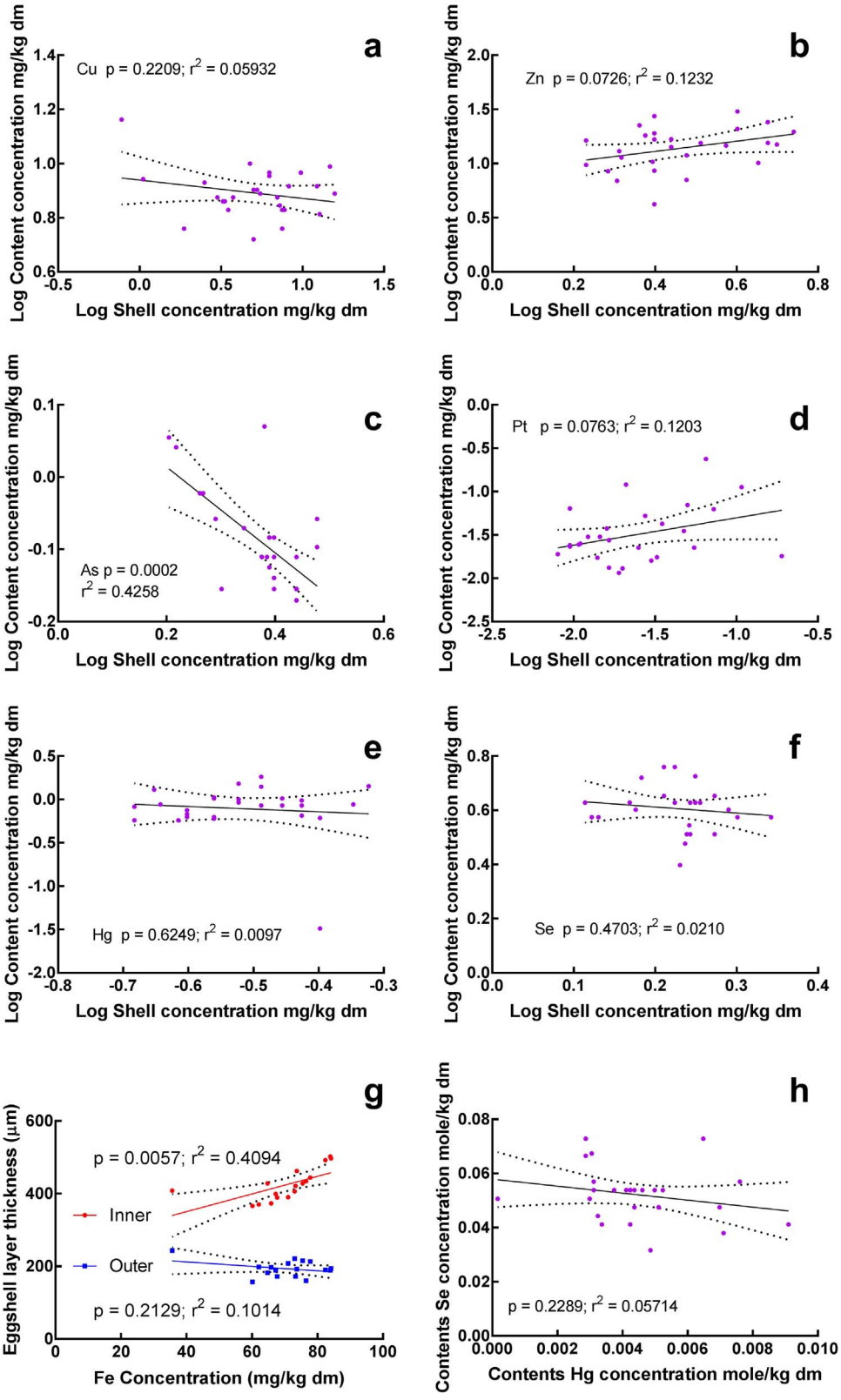
a
Cu p = 0.2209; r2 = 0.05932
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
b
Zn p = 0.0726; r2 = 0.1232
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
c
As p = 0.0002
r2 = 0.4258
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
d
Pt p = 0.0763; r2 = 0.1203
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
e
Hg p = 0.6249; r2 = 0.0097
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
f
Se p = 0.4703; r2 = 0.0210
Log Content concentration mg/kg dm
Log Shell concentration mg/kg dm
g
p = 0.0057; r2 = 0.4094
Inner
Outer
p = 0.2129; r2 = 0.1014
Eggshell layer thickness (µm)
Fe Concentration (mg/kg dm)
h
p = 0.2289; r2 = 0.05714
Contents Se concentration mole/kg dm
Contents Hg concentration mole/kg dm

Fig. 2. Linear regressions and associated statistics of elemental concentrations in eggshells and egg contents (2a-f). Linear regression of iron against inner eggshell thickness (2 g). Linear regression of molar concentrations of selenium and mercury in egg contents (2 h).

Gorge and Nhlanganini Dam egg contents, but Cu, Ag, Ba, and Pb differed between the closely located Gorge and Letaba collection areas (Table 2 and Fig. 1). There were also relatively few differences between the Gorge and Nhlanganini Dam shells, and more between Letaba and Gorge (however, note the difference in Hg - this difference was not seen in the contents). Because there were relatively few differences and that the absolute means did not differ by any order of magnitude, it seems therefore that female Nile Crocodiles manage to regulate elemental compositions in both shells and contents. This is further borne out by the few significant differences when the wild crocodile eggs and Crocodile Farm eggs are compared. For wild crocodiles, general geological background, wide-ranging movement of the females, and up- and

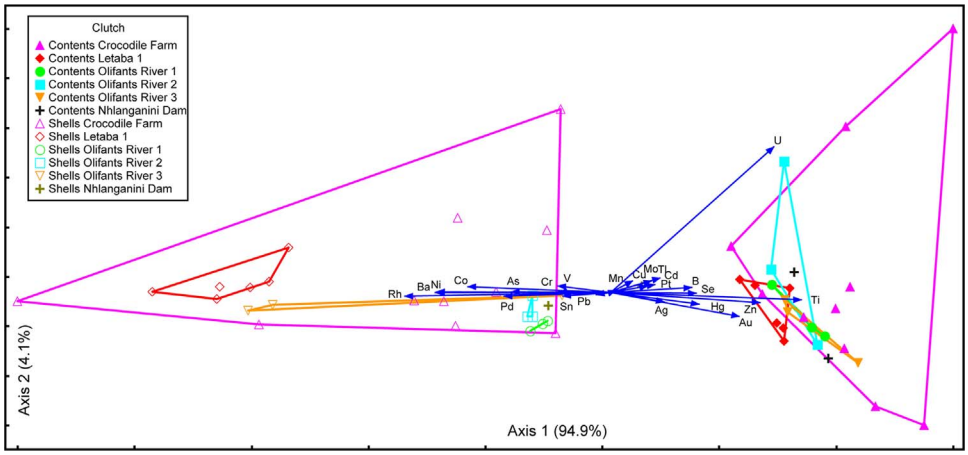

Fig. 3. Non-metric multidimensional scaling ordination plot of relativized elemental concentrations in eggshells and contents. Iron was not included as its relativized concentrations in shells were very high, compared with the contents. Convex hulls encompass individual clutches. The final instability was 0.0000, and the final stress was 3.828.

downstream movement of prey fish (Cambray, 1990) may have evened out the uptake differences over the lifetimes, but this was not the case for the Crocodile Farm crocodiles being fed chicken and severely restricted in movement. Since the Olifants River, Letaba River, and Nhlanganini Dam areas are all linked (Fig. 1) it may be that a relatively homogenous elemental composition of the fish population is in play.

A number of studies have focused on metals and metalloids in fish associated with the KNP (e.g. du Preez et al., 1997; Coetzee et al., 2002; Dabrowski and de Klerk, 2013). None of the elemental concentrations (in crocodile egg contents) listed in Table 4 for which we could find comparable fish data showed notable elevated concentrations. Although there are wide differences in concentrations reported (most notably Fe), no pattern that could explain or inform why the concentrations in crocodile eggs stood out. More studies are needed to elucidate biomagnification if crocodiles do biomagnify of some elements via trophic transfer.

4.2. Multivariate analyses

The relative elemental composition (fingerprints) of the egg contents and shells for each clutch confirms a greater homogeneity of the egg contents (smaller convex hulls) compared with the shells that had relatively larger convex hulls (Fig. 3). The convex hulls of the Crocodile Farm samples were much larger than expected, partially influenced by the high relative U composition of one egg and shell (17 and 28 mg/kg dm, respectively, for the top right of both shell and content hulls in Fig. 3). The large convex hulls for both shells and contents for Crocodile Farm belie the expectations that a captive environment, and constant feed and geological background would result in a compact (small) convex hull when compared with wild crocodiles.

For wild crocodile eggs however, the convex hulls of the contents all overlapped (Fig. 3), supporting a homogenised background in food, geology, and physiological regulation (Fig. 3). For the convex hulls of wild shells, apart from having a very different relative elemental composition compared with contents, surprisingly, did not overlap, suggesting less physiological regulation.

Essential elements may be regulated better than non-essential elements and therefore can be expected to have smaller %CVs (Grillitsch and Schiesari, 2010). The mean percentage coefficient of variation of the elements (Table 3) in shells was somewhat higher than in the contents (42 vs 37). The mean %CV of the shells and contents did not differ significantly (Wilcoxon matched-pairs, signed rank test), but the pairing was effective, indicating that elements with high variation in one matrix tended to have higher variation in the other, and vice versa. Vanadium and Cr had small %CVs in shells and contents respectively, while Au and U had the largest, up to 263%. It seems therefore, that some of the elements are more effectively regulated than others.

Shells were strongly associated with higher proportional Ni, Co, Ba, Rh, and As (Fig. 3). Iron was not included in the ordinations because of its very high concentrations in shells, which was also high compared with other data (Table 1). High concentrations of Cr, As, Fe, Ni and Co were reported (Gerber et al., 2015b) in the sediment of both the Letaba and Olifants Rivers, near the vicinity where the eggs were collected. The leathery shells of crocodile eggs are semi-permeable, and may allow migration of bio-available elements into and out of the shell (Marco et al., 2004; Guirlet *et al.*, 2008). The wild eggs were in the sand for some time and autochthonic uptake of elements from the sand into the eggshell could have taken place (see Grillitsch and Schiesari, 2010 for an overall discussion on contrasting findings, especially concerning Nagle et al., 2001 and Sahoo et al., 1996). However, the similarity of elemental composition between the Crocodile Farm egg contents and shells with the wild-collected samples precludes this possibility (at least within the parameters of our study), as the farmed eggs were collected within days of being laid and placed in incubators, reducing the time available for autochthonic uptake of elements from the sand. It also seems that mercury is not taken up from the ambient substrate by eggs of the Colombian Slider Turtle (*Trachemys callirostris*; Rendón-Valencia et al., 2014). Still, autochthonic uptake cannot be entirely excluded from this study and needs further investigation.

4.3. Behaviour and feeding

Nile Crocodiles have winter basking and summer nesting grounds (Huchzermeyer, 2003; Combrink et al., 2017). Using mobile biota as indicators of accumulated pollutants in areas where movement is not restricted only gives an indication of the lager area's state of pollution (Gerhardt, 2007; van der Schyff et al., 2016). Differences in egg elemental compositions between crocodiles from different regions may be ascribed to diverged preferences for food types and availability. The crocodiles from the Gorge prey mostly on fish because the gorge is steeply sloped, restricting the access of larger mammals. The Olifants River contains a variety of fish species on which crocodiles thrive (There are four recognised Fish Sanctuaries within 10 km radius of OL2 collection site. Fig. 1; SANBI, 2017). At Nhlanganini Dam, both mammalian wildlife and fish are available as food (the confluence of the Nhlanganini and Letaba rivers is also a Fish Sanctuary; SANBI, 2017; Fig. 1). In the Okavango Delta, Botswana, the major prey items for larger crocodiles was also found to be aquatic, mostly fish, despite the availability of large vertebrates (Wallace and Leslie, 2008). We may there assume that the dominant source of prey for KNP crocodiles would be aquatic as well.

Many studies have focused on metals and metalloids in fish in the Olifants River (e.g. du Preez et al., 1997; Coetzee et al., 2002; Dabrowski and de Klerk, 2013). There is some information available on metals in terrestrial wildlife in KNP. A copper mine is located just outside the KNP at the town of Phalaborwa (Fig. 1). Cattle deaths near

Table 4
Summary of elemental concentrations in fish from the Olifants and Letaba rivers from other studies.

Species	Sample	Year	Matrix	Locality, all South Africa	Al	Cu	Pb	Cr	Ni	Co	Cd	Zn	Se	As	Mn	Fe	Ba	V	Ag	Reference
Nile Crocodile	**Dry**	**2009**	**Content**	**Olifants Gorge, KNP**	**16**	**7.7**	**0.76**	**1.5**	**0.48**	**0.045**	**0.018**	**14**	**4.6**	**0.74**	**1.7**	**52**	**7.8**	**0.51**	**0.13**	**This Study**
Tigerfish	Dry	2010	Muscle	Olifants River, KNP	4.1	0.37	0.013	0.15	0.29	0.0062	0.028	20	1.4	0.086	0.33	44				Gerber et al. (2016)
Tigerfish	Dry	2009	Muscle	Olifants River, KNP	28	1.9	0.16	0.55	0.43	0.075	0.068	23	0.82	0.27	1.6	55				Gerber et al. (2016)
Sharptooth Catfish	Dry	1994	Muscle	Olifants River, KNP		4.6		68								1700				Oldewage and Marx (2000a)
Sharptooth Catfish	Dry	1994	Muscle	Olifants River, KNP					53						13					Oldewage and Marx (2000b)
Sharptooth Catfish	Dry	1994	Muscle	Olifants River, upstream KNP		2						31								Kotze et al. (1999)
Mozambique Tilapia	Dry	1994	Muscle	Olifants River, KNP		8.1		84							17	1800				Robinson and Oldewage (1997)
Mozambique Tilapia	Dry	1994	Muscle	Olifants River, KNP		1.0						41								Kotze et al. (1999)
Mozambique Tilapia	Dry	2008	Muscle	Olifants River, upstream KNP	59	4.9	4.8	14	4.9	0.4	0.2	210	2		15	130	300	1.9	5.3	Addo-Bediako et al. (2014)
Nile Crocodile egg	**Dry**	**2009**	**Content**	**Letaba River KNP**	**20**	**6.6**	**0.58**	**1.4**	**0.48**	**0.048**	**0.020**	**12**	**3.7**	**0.8**	**2**	**55**	**10**	**0.48**	**0.021**	**This Study**
Tigerfish	Dry	2010	Muscle	Letaba River, KNP	1.8	0.88	0.044	0.16	0.35	0.027	0.07	39	2.3	0.071	2	52				Gerber et al. (2016)
Nile Crocodile egg	**Dry**	**2009**	**Content**	**Nhlanganini, KNP**	**19**	**9.6**	**1.5**	**1.5**	**0.44**	**0.049**		**15**	**3.8**	**0.71**	**1.6**	**52**	**7.2**	**0.55**	**0.32**	**This Study**
Sharptooth Catfish	Dry	1994	Muscle	Nhlanganini Dam, KNP		1						46								Kotze et al. (1999)
Mozambique Tilapia	Dry	1994	Muscle	Nhlanganini Dam, KNP		1						28								Kotze et al. (1999)

Phalaborwa were ascribed to chronic copper poisoning from copper deposited on vegetation (Gummow et al., 1991). Macropathology found excess body fluids in the body cavities and pericardial sacs, enlarged friable livers, congested spleens and kidneys, and haemoglobinurea were all consistent with copper poisoning. Liver and kidney histopathology also showed effects from haemolysis, ascribable to excess copper intake (Gummow et al., 1991). Cape Buffalo *Syncerus caffer* and Impala *Aepyceros melampus* inside the KNP and adjacent to the copper mine had significantly higher concentrations of copper then elsewhere in the KNP (Gummow et al., 1991; Grobler and Swan, 1999). Two dead Impala inside the KNP near Phalaborwa had very high concentrations of copper. Other Impala and Cape Buffalo also had very high concentrations inside and outside the KNP, with significantly higher mean concentrations near the mine (Gummow et al., 1991; Grobler and Swan, 1999). An associated study on Impala from the same areas found increased sperm damage significantly associated with elevated copper (Ackerman et al., 1999), indicating the effects that pollutants originating outside reserves may eventually have on populations of mammals and reptiles in protected areas (Köhler and Triebskorn, 2013). The crocodile egg and eggshell concentrations we found were higher than for any wild samples from anywhere else (USA, China; Table 1), raising concerns.

For crocodilians, a non-food route of uptake of metals and metalloids is also possible. Crocodilians ingest sediment and mud while feeding, as well as accidentally or purposely ingesting pebbles (lithophagy) as gastroliths that may aid in digestion and/or act as ballast (Huchzermeyer, 2003; Grillitsch and Schiesari, 2010). The acidic stomach fluids (with a pH as low as 1.2; Huchzermeyer, 2003) may therefore liberate minerals and elements from inorganic substrates that then becomes available for uptake. That such may happen was illustrated by high lead concentrations in Nile Crocodile blood, most likely derived from ingested lead sinkers (Warner et al., 2016).

4.4. Comparisons with other crocodile egg data

The higher than comparable Al concentrations (Table 1) in crocodile contents and shells from the KNP may have been caused by wrapping the eggs in aluminium foil upon collection. However, uptake of aluminium from foil is not a definitive explanation and awaits future research opportunities, especially given the concerns expressed by Oberholster et al. (2012) about the possible involvement of aluminium in the crocodile deaths in the KNP.

Lead in contents was lower than concentrations reported from Zimbabwe (Phelps et al., 1986) but noticeably higher than crocodile and alligator eggs from other continents. Copper concentrations in KNP egg contents and shells were higher than anywhere else. Cadmium was about the same or less, while Zn had lower concentrations than in Zimbabwe but higher compared with most other reports. Cobalt was low, but there was only one datum to compare with (Table 1).

Mercury concentrations found in this study were higher in contents and shells than from almost anywhere else, as were Cr, Se, As, Mn, and Fe concentrations in KNP egg contents (Table 1). Based on these findings, continued scrutiny of toxic metals in crocodile eggs is called for in the KNP and elsewhere in Africa due to increased human population and industrial activities.

4.5. Iron and eggshell thickness

The concentrations of Fe in crocodile eggshells from the KNP are comparably high (Table 1). The influence on crocodile eggshell properties of high concentrations of Fe is not known. However, there is some (contradictory) information from supplementary iron feeding studies on chicken eggs. Organic iron supplementation (iron-soy proteinate and iron-methionine chelate) deepened eggshell colour but did not affect eggshell strength or thickness (In-Knee et al., 2009). In a similar feeding study, organic iron supplementation increased eggshell strength and

colour (thickness not reported), but the greatest effect on strength was by an inorganic iron supplement - $FeSO_4$ (Park et al., 2004). Break strength increased from 52 to 60 kg/cm^2 - about 15%. If we assume that the eggshell thickness of crocodilians do not change during incubation (as was found for the American Alligator; Ferguson, 1981), any change in eggshell thickness and/or strength due to action by pollutants might therefore affect embryo development, survival, and hatching.

In the study we completed on POPs in the same eggs reported here (Bouwman et al., 2014l), we found indications of eggshell thickening (of the outer layer) associated with DDE in the Gorge shells. Others working with crocodilian eggs found similar or contradictory evidence with POPs (see discussion in Bouwman et al., 2014). Gorge shells also had the highest Fe concentration (mean 1900 mg/kg dm; Table 1). Linear regressions of inner and outer eggshell thickness of all eggs against Fe showed a marginally significant ($p = 0.0783$) thinning of the outer layer, and a marginally thickening of the inner layer ($p = 0.0755$). However, for the wild eggs only, the inner thickness increased significantly with increased Fe ($p = 0.0057$; $r^2 = 0.4094$; normally distributed, untransformed data; Fig. 2g) from 0.366 to 0.502 mm. Removing the shell with the lowest Fe concentration (far left in Fig. 2g - which was one of the two from Nhlanganini Dam) resulted in $p < 0.0001$ and $r^2 = 0.8158$. The thickest inner-shell was about 37% thicker than the thinnest. The outer layer thickness did not change significantly.

Thicker shells (with the possible additional complication of a shell stronger due to increased iron content) may affect hatchability. Eggs with “abnormally” thickened shells outer shells caused ‘early embryonic death’ in American Crocodile (Wink and Elsey, 1994). Thicker shells may also affect water and gas exchange, although the alligator egg membranes (which are much thicker than in bird eggs) are largely determining gas exchange (Kern and Ferguson, 1997). For reptile eggs that have no air pockets, a thicker shell may however be more important. Clearly more work is needed on the effects of different minerals and organic pollutants on changes in reptile eggshell strength and possible thickening, as well as using the shell to deposit excess or toxic chemicals.

4.6. Toxicity

Definitive cause-effect relationships between chemical agents and biota are very difficult to establish outside of controlled laboratory conditions due to multiple environmental exposures and, for the larger reptiles, long lifespans where effects of previous exposures may be expressed at a later age when traces or evidence of initial exposure are difficult to find. However, early live stages are sensitive to xenobiotic chemical action by affecting viability and development (Grillitsch and Schiesari, 2010; Köhler and Triebskorn, 2013). There are several ways to investigate whether elements may be deleterious at certain concentrations in the absence of taxon-specific exposure-effects studies. One is a comparison with reported effects from field observations, and the other is using levels of concern derived for other taxa.

The concentrations of Pb, Ni, and Co were high (compared with our results in Table 1) in American Alligator eggs from Florida Bay where reproductive deformities were reported (Stoneburner and Kushlan, 1984). We are not aware of any deformities reported from the KNP, although it is likely that handicapped hatchlings would be easily predated and therefore not observed.

Mercury is known to cause neurological and reproductive deformities (Dietz et al., 2013; Hopkins et al., 2013). The Hg concentrations in the wild crocodile egg contents from KNP were slightly higher than from elsewhere (Table 1), but remarkably lower in eggshells from most other sites. The closest relevant toxicological study we could find concerned the Common Snapping Turtle (*Chelydra serpentine*; Hopkins et al., 2013). Eggs collected along a mercury pollution gradient of a river in Virginia, USA, showed a significant decrease in hatching success and significant increases in embryonic mortality and frequency of unfertilized eggs with an increase in Hg concentrations (0.01 − 6.6 mg/kg dm in contents). These concentrations are of the same order of magnitude as our findings (Table S1; maximum concentration of 1.8 mg/kg dm in a Crocodile Farm egg, and 1.3 mg/kg dm in a wild egg). In a previous study on metals in crocodile muscle tissue from the KNP (du Preez et al., 2016), we also identified mercury as a cause for concern. The bird egg-based toxic reference value (TRV) for mercury in bird eggs is 2 mg/kg dm (Meyer et al., 2014). Assuming valid cross-taxon extrapolation, the maximum concentration in a wild egg from the KNP did not reach this level. However, there remains a well-justified concern and further investigation is warranted.

The TRV for copper is between 10–20 mg/kg dm, which was reached and exceeded in Crocodile Farm and wild eggs (Table S1). Copper may therefore also pose a threat to developing crocodiles. The TRV for Se is 8 mg/kg dm - the highest concentration we found in a wild crocodile egg was 5.8 mg/kg dm (Table S1). Selenium therefore may also pose a risk to the developing embryo. Since Se seems to be involved in the detoxification of Hg in Leatherback Turtles *Dermochelys coriacea* (Perrault et al., 2011), we regressed the molar concentrations of these two elements in egg contents (Fig. 2h) but found no association. Selenium concentrations remained relatively constant, as would be expected from an element that is regulated physiologically.

4.7. Eggshells as proxy for contents?

It would be advantageous to use eggshells as representative tissue only, thereby minimising the impacts on populations. However, as indicated in Figs. 2 and 3, in Table 2 and S2, as well as the MRPP results (all interpreted above), using shells as proxy for contents would not be suitable. Some shell elemental concentrations had no association with content concentrations, some were positive, and some negative.

4.8. Synthesis and conclusions

Reptiles are under-represented in ecotoxicological literature (Grillitsch and Schiesari, 2010). To the best of our knowledge, this is the first report of metallic elements in crocodile eggs from South Africa, and only the second from Africa in 30 years. There are five publications on metallic elements in Nile Crocodile tissues other than eggs (Hoffman et al., 2000; Swanepoel et al., 2000; Almli et al., 2005; du Preez et al., 2016; Warner et al., 2016). The three that measured mercury all concluded that this element is of concern (Swanepoel et al., 2000; Almli et al., 2005; du Preez et al., 2016). There are also very few toxicological publications on other freshwater, marine, or terrestrial reptiles from Africa. The findings in Zimbabwe (Phelps et al., 1986) and those presented here, indicates a large gap in our understanding of the concentrations and threats of metals and metalloids in an important class of animals in Africa. We have identified at least Hg, Se, and Cu as metals of concern. Mercury and Cu are waste-, industrial- and mining-related and this concern should therefore be extended to all areas where the four, currently recognised, African crocodile species occur. [The other species are the Slender-snouted crocodile *Crocodylus cataphractus*, West African Crocodile *Crocodylus suchus*, and the Dwarf crocodile *Osteolaemus tetraspis* http://www.iucncsg.org/pages/Crocodilian-Species.html.]

We also identified Fe as a possible contributor to thickening of eggshells as a barrier to gas and water exchange, possibly increasing the effort required for the hatchling to emerge from tightly packed shells under sand or nesting materials. Other effects in reptiles associated with metals include haematological, immunological, genetics, neurological, and developmental effects, endocrine disruption, and effects on behaviour that may all reflect eventually on populations and ecosystems (Grillitsch and Schiesari, 2010; Köhler and Triebskorn, 2013), including the river systems of the KNP. Of major concern is the effect of pollutants on long-lived animals, such as crocodiles. Reduced fitness may only become apparent quite late in animals with decadal lifespans as the

parents remain obviously present, but the reduction in numbers of the less obvious young could mask changes in age structure. Not only are the direct effects on the parental generation of concern, but also the effects of exposure to pollutants starting with embryonic development that may affect populations decades after initial exposure, especially for larger, long-lived reptiles such as crocodiles and sea turtles. Rowe (2008) provides an excellent discussion on this topic.

We have sampled only two of the six major river systems of the KNP. Crocodile numbers in the northern Levuvhu River (to the far north of the KNP) have also declined (Ferreira and Pienaar, 2011). Since we sampled relatively few eggs for this study (given the concern at the time of impacts on the various populations), it would be opportune to collect, analyse and interpret organic and inorganic residue data for more crocodile eggs from more rivers, now that concerns have been established. Although there is reasonable knowledge on the general biology of the Nile Crocodile and the other three crocodilians in Africa (e.g. Calverley and Downs, 2014; Shirley et al., 2016; Calverley and Downs, 2017; Zisadza_Gandiwa et al., 2013), there is very little known about hatching success apart from nest predation (Combrink et al., 2016; Calverley and Downs, 2017).

Considering the increasing international focus on mercury (The Minamata Convention on Mercury; http://www.mercuryconvention.org/), and recognising that the major river systems of the KNP are trans-boundary (as are most major river catchment systems in Africa), underscores the need for further research on the biology and ecotoxicology of all African reptiles and associated habitats. Habitat destruction, pollution, human population increases, and climate change all are interacting factors that will affect how African reptiles will cope. The Nile Crocodile is the largest predator in the KNP and in Africa. The latest available census indicates 4 420 individuals (KNP, 2011). The rivers that carry water from outside the park sustain its aquatic life that includes the Nile Crocodile, but also transport pollutants into the Park. Hence, improvements in source mitigation remains an important task and responsibility for all involved.

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Conflicts of interest

None

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ecoenv.2017.11.032.

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